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1 Surfactant TWEEN20 provides stabilisation effect on anthocyanins extracted
2 from red grape pomace

3
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12
13 **Abstract**

14 Red grape pomace, a wine-making by-product is rich in anthocyanins and has many
15 applications in food and pharmaceutical industry. However, anthocyanins are unstable
16 during processing and storage. This study aimed to investigate the stability of anthocyanins
17 obtained by hydroalcoholic extraction (with and without sorbic acid) and colloidal gas
18 aphrons (CGA) separation; a surfactant (TWEEN20) based separation. Anthocyanins in
19 CGA samples showed higher stability (half-life= 55 d) than in the crude extract (half-life=
20 43 d) and their stability increased with the concentration of TWEEN20 in the CGA fraction
21 (6.07-8.58mM). The anthocyanins loss in the CGA sample (with the maximum content of
22 surfactant, 8.58 mM) was 34.90%, comparable to that in the crude ethanolic extract with
23 sorbic acid (EE-SA) (31.53%) and lower than in the crude extract (44%). Colour stabilisation
24 was also observed which correlated well with the stability of individual anthocyanins in the
25 EE and CGA samples. Malvidin-3-o-glucoside was the most stable anthocyanin over time.

26
27 **Keywords:** Grape pomace, anthocyanins stability, colloidal gas aphrons, surfactant, storage

28 *Abbreviations:* ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AOP,
29 antioxidant power; CGA, colloidal has aphrons; EE; ethanolic extract, EE-SA, ethanolic
30 extract with addition of sorbic acid; GAE, gallic acid equivalents; $t_{1/2}$, half-life; glc, 3-o-
31 glucoside; ME, malvidin glucoside equivalent; V4, CGA fraction separated at volumetric
32 ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at
33 volumetric ratio 16.

34

35 **1. Introduction**

36 Grapes are one of the most important fruit crop cultivated across the world, whereby
37 80% of the grape productions are used in wine-making industry (Fontana, Antoniolli, &
38 Bottini, 2013). Wine production is considered one of the most important agricultural
39 activities, generating large amount of residues including grape skins, stems and seeds (Yu &
40 Ahmedna, 2013). At the end of the fermentation process, large amounts of resiudes are being
41 discharged containing high amount of phenolic compounds including anthocyanins,
42 catechins, flavonol glycosides, phenolic acids and stilbenes (Kammerer, Kammerer, Valet,
43 & Carle, 2014). This is seen by the environmental management authorities as a serious threat
44 because they are low in pH and high in organic matter thus potentially causing a phytotoxic
45 effect if applied to crops or wetlands (Kammerer, Claus, Carle, & Schieber, 2004; Lavelli,
46 Harsha, Laureati, & Pagliarini, 2017). Therefore, converting and utilising this by-product to
47 another useful product would be a solution to this problem. For instance, the anthocyanins
48 from this pomace can be used as natural food colourant (Thakur & Arya, 1989).
49 Anthocyanins are sensitive to thermal degradation making the recovery rather difficult and
50 complex, but they are on demand due to their wide applications in food (already being used
51 as food colourants, E163, approved by EC) as well as in pharmaceuticals and cosmetics.
52 Thus, various extraction techniques have been studied and used, including acidified alcohol,
53 sub- and supercritical fluid and high pressure processing (Barba, Zhu, Koubaa, Sant'Ana, &
54 Orlie, 2016; Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015;
55 Lozovskaya, Brenner Weiss, Franzreb, & Nusser, 2012).

56 Food processing generally involves thermal processing prior to consumption and this
57 process has a great influence on the anthocyanins content in the final product. Thermal
58 processing involves high temperatures ranging from 50°C to 150°C, depending on the pH
59 and the desired shelf life of the product. Anthocyanins are expected to degrade over time.
60 However, the storage temperature has been found to be an important factor that is affecting
61 anthocyanins' shelf life. Degradation of anthocyanins is greatly affected by the type of
62 anthocyanin, the origin of the samples and the storage temperature (Hellström, Mattila, &
63 Karjalainen, 2013). The thermal degradation of anthocyanins in extracts and model systems
64 are reported to follow first-order reaction kinetics (Presilski, Presilska, & Tomovska, 2016).

65 The stability of anthocyanins can be improved, by self-association of the anthocyanins,
66 removal of oxygen and inactivation of enzymes (Hellström et al., 2013). In the food
67 industry, the sensitivity of bioactive compounds is addressed by incorporating edible
68 coatings as a structural matrix, used widely to create a barrier from oxygen, moisture and
69 solute movement (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Encapsulating
70 methods such as spray drying/spray chilling or liposomes have been used. The former
71 requires liquid droplets or small particles being incorporated within a continuous edible
72 coating, thus it requires an emulsifier. Liposomes are microscopic spherical particles
73 consisting of one or more lipid bilayers that can encapsulate or bind a variety of molecules.
74 Therefore, particularly in food applications, food grade surfactants such as TWEEN20 have
75 been used as emulsifying agents to fit this purpose (Quirós-Sauceda, Ayala-Zavala, Olivas,
76 & González-Aguilar, 2014). Moreover, TWEEN20 has been seen as having a profound
77 protective effect on five different polyphenols, by slowing down the auto-oxidation process
78 at pH 4.5 (Lin, Wang, Qin, & Bergenståhl, 2007).

79 A surfactant-based separation technique, colloidal gas aphrons (CGA) has been
80 previously studied in our group to recover various valuable bioactive compounds from

different feedstock such as astaxanthin (Dermiki, Bourquin, & Jauregi, 2010; Dermiki, Gordon, & Jauregi, 2009), proteins (Fuda & Jauregi, 2006; Fuda, Bhatia, Pyle, & Jauregi, 2005) and polyphenols (MohdMaidin, Michael, Oruna-Concha, & Jauregi, 2017; Spigno, Dermiki, Pastori, Casanova, & Jauregi, 2010; Spigno, Amendola, Dahmoune, & Jauregi, 2015). The type of surfactant (i.e cationic, anionic and non-ionic) determines the outer charge of the CGA, where molecules with the opposite charge will attract to the CGA resulting in their effective separation into the CGA phase.

In our previous work it was shown that 70% of the anthocyanins could be recovered from the ethanolic extract of grape pomace using CGA generated from TWEEN20. The CGA fraction will be rich in surfactant therefore, it will be interesting to test what will be the added value of extracting the anthocyanins in such a solution and whether this can offer any advantage to their formulation for subsequent applications. Thus the present study aimed at assessing the stability of anthocyanins in the CGA separated fraction over time in comparison with their stability in the crude ethanolic extract (EE) (before the CGA separation) as well as in the crude ethanolic extract with a commercial additive, sorbic acid (EE-SA). It is therefore hypothesised that the anthocyanins in the CGA sample will show higher stability than in the crude extract over time.

2. Materials and methods

2.1 Materials

Grape pomace (Barbera variety) was obtained from a winery in Northern Italy. All the solvents (purity of 95% and above) used in this project were obtained from Sigma-Aldrich Company Ltd., Dorset, UK. For the HPLC analysis, the solvents used were of HPLC grade (purity of 98-99.9%) also from Sigma Aldrich.

105 2.2 *Extract preparation*

106 The grape pomace (Barbera variety) was kindly provided by a winery located in
107 Northern Italy. At the winery, the fermented pomace was recovered and oven dried at 60°C
108 until the residual moisture content is <5%. The dried pomace powder was sieved with a 5mm
109 sieve to separate the skins from the seeds and milled into fine powders with particles size <
110 2mm and stored in the freezer at -20°C until further use.

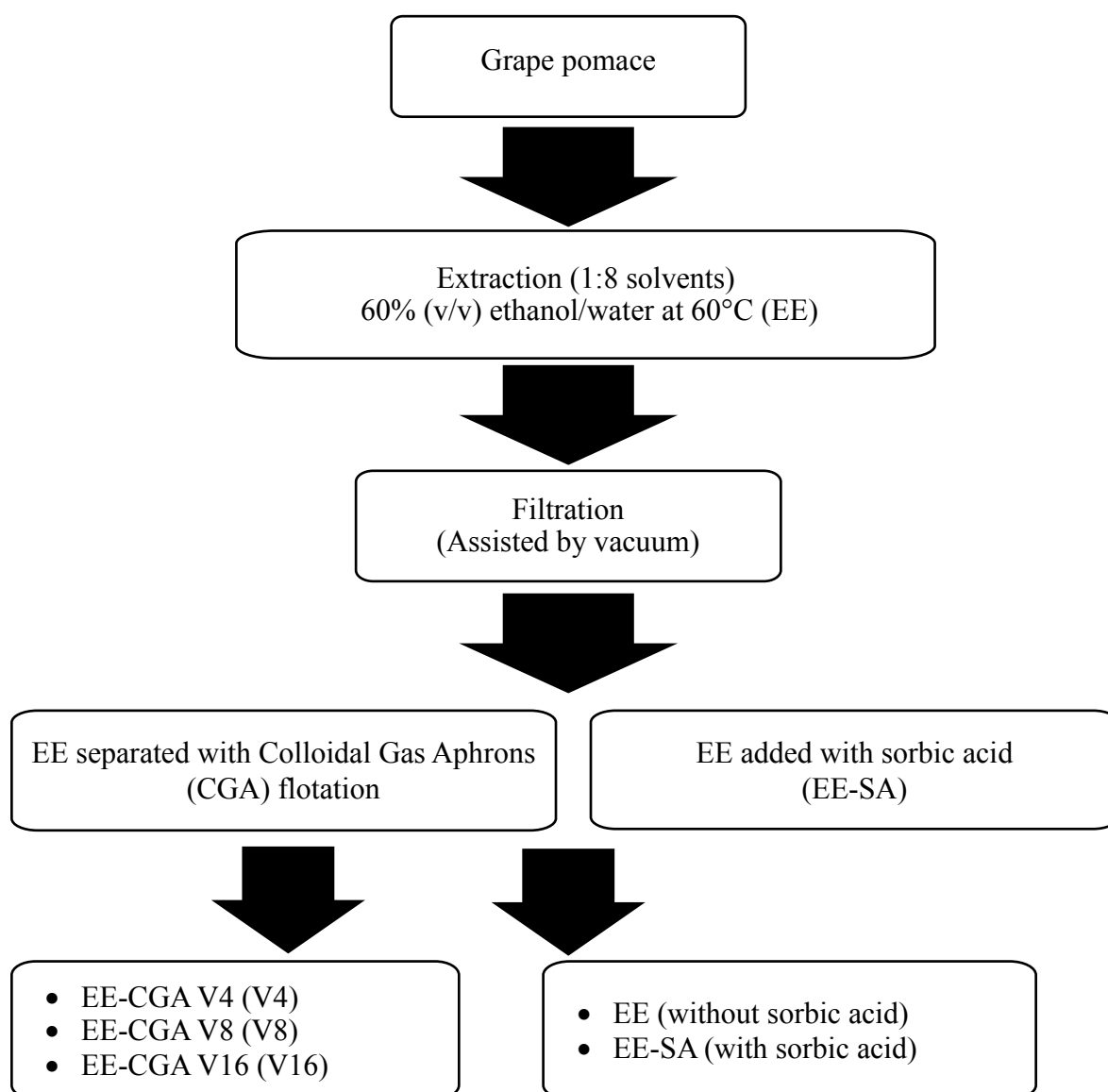
111 The extraction procedure was done in accordance to our previous study using ethanol-
112 aqueous solvent (MohdMaidin et al., 2017). The extract was filtered and two different
113 samples were produced: (1) approximately, 400mL of the ethanol extract labeled as EE and
114 (2) another 400mL ethanol extract with sorbic acid (>99%) (Sigma, UK) and labelled as EE-
115 SA. Sorbic acid was chosen in this study for its wide application as food additive, thus
116 making it closer to the formulation of most low pH food products and neutral taste (Troller
117 & Olsen, 1967). Both EE and EE-SA were considered control samples. The remaining
118 filtrate of 800mL was kept aside for CGA separation.

119

120 2.3 *CGA separation using 10mM TWEEN20*

121 The separation of polyphenols from the crude ethanolic extract was carried out at
122 different volume ratios of CGA to feed (V_{CGA}/V_{feed}). The ratios selected were 4, 8 and 16.
123 The separations were individually carried out in a flotation glass column according to the
124 method described in our previous work (MohdMaidin et al., 2017); each separation was
125 carried out in triplicate. It should be noted that as the volumetric ratio increased, so did the
126 concentration of TWEEN20 in the solution of the separated CGA fraction. The concentration
127 of TWEEN20 in each of these fractions was estimated from a knowledge of the separated
128 volume of CGA and corresponding liquid fraction which was determined from a
129 measurement of gas hold-up (gas volumetric ratio defined as the volume of air incorporated

130 in a given volume of CGA dispersion) of the CGA generated with this solution of TWEEN20
 131 (61.3%). The estimated concentrations were: in V4, 6.07mM, in V8, 7.56mM and in V16,
 132 8.58mM. The summary of the extraction and separation process is briefly described in Figure
 133 1.
 134



135 * All samples were stored at 20°C for 32 days

136 Figure 1: Flow diagram of hydroalcoholic extraction and CGA separation processes applied
 137 to grape pomace, n = 3; EE is the ethanolic extract; EE-SA is the ethanolic extract with
 138 addition of sorbic acid; EE-CGA V4 is the ethanolic extract further processed with CGA at
 139 CGA to feed volumetric ratio of 4; V8 and V16 correspond to the extracts further processed
 140 with CGA at CGA to feed volumetric ratios 8 and 16 respectively

141 2.4 Determination of degradation of chemical and physical properties over time

142 Briefly, the EE, EE-SA and CGA fractions were divided in equal volumes and kept in
143 sterilised containers in the darkness. These were then stored at room temperature 20°C (SD
144 1°C) which was regularly monitored using a thermometer for 32 days. The total phenolic
145 content, total anthocyanin and antioxidant activity were determined as described in section
146 2.5-2.7. The total anthocyanins, individual anthocyanins, antioxidant capacity and the colour
147 degradation over time (32 days; every day for the first 7 days and subsequently 5 days
148 intervals) were determined.

149 The kinetics of degradation of total anthocyanins and individual anthocyanins were
150 assessed; the natural logarithms of these were plotted against time in order to test for first-
151 order kinetics as described by the equation below:

152

$$153 \quad -\ln\left(\frac{A_t}{A_0}\right) = k * t \quad (\text{Equation 1})$$

154

155 Where A_0 is the initial anthocyanin content, A is the anthocyanin content at time t , t is the
156 storage time and k is the rate constant. The degradation rate constant (k) was determined
157 from the slope of the straight line obtained when plotting $\ln(A_t/A_0)$ vs t . From the equation
158 above, the time taken for the anthocyanin content to halve, the half-life ($t_{1/2}$), can be derived
159 as:

160

$$161 \quad t \frac{1}{2} = \frac{\ln(2)}{k} \quad (\text{Equation 2})$$

162

163 2.5 *Total phenolic content*

164 Folin Ciocalteu (FC) colorimetry method (Singleton & Rossi, 1965) was employed to
165 determine the total phenolic content of the EE and EE-SA (control samples) and also in all
166 of the CGA processed samples. This method involves the oxidation of phenols using a
167 molybdotungstate reagent to yield a coloured product which can be measured at 760nm using
168 a spectrophotometer (Biotech Ultrospec 1100 pro UV spectrophotometer). Gallic acid
169 (Sigma-Aldrich, UK) standards with concentrations ranging from 0-1000mg/L were used to
170 generate standard plots ($R^2 = 0.9881$) and an equation for the calculation of the total phenolic
171 concentration in each extract. The analysis was done in triplicate. The total phenolic content
172 in the CGA processed samples were compared to the controlled samples over time.

173

174 2.6 *Evaluation of in vitro antioxidant activity*

175 The antioxidant activity of the control samples (EE and EE-SA) along with the CGA
176 processed samples were evaluated according to Re et al., (1999) by the ABTS assay. This
177 method assesses the ability of the antioxidants to scavenge the radical (ABTS) which was
178 determined by measuring the decrease in its absorbance at 734nm using a spectrophotometer
179 (Biotech Ultrospec 1100 pro UV spectrophotometer). Different concentrations (0-2000 μ M)
180 of Trolox standard were used to construct a calibration curve ($R^2 = 0.9991$). The analysis was
181 done in triplicate. The antioxidant activity of the CGA processed samples was compared
182 against that of the control samples, expressed as μ M Trolox equivalent. The ratio of
183 percentage inhibition to the total phenolic content of all samples, termed as specific
184 antioxidant power (AOP), was calculated.

185

186 2.7 Total anthocyanins content

187 The total monomeric anthocyanins of control samples, EE and EE-SA along with the
188 CGA processed samples were determined over time using the pH differential method
189 approved by AOAC (Lee, Rennaker, & Wrolstad, 2008). This method is based on the
190 anthocyanins structural transformation that occurs with a change in pH. Briefly, the extract
191 was mixed individually with pH 1.0 and 4.5 buffer solutions in a ratio of 1:5 and left for 20
192 minutes. The absorbance of the test portions at both pHs were determined
193 spectrophotometrically (Biotech Ultrospec 1100 pro UV spectrophotometer) at a wavelength
194 of 520nm and 700nm. The results of the anthocyanin pigment were expressed as malvidin-
195 3-glucoside equivalents (ME) according to equation 3.

196

$$197 \quad Total\ Anthocyanins \left(ME, \frac{mg}{L} \right) = \frac{A * MW * DF * 10^3}{\epsilon * 1} \quad (Equation\ 3)$$

198

199 Where A = (A_{520nm}-A_{700nm})_{pH1.0} - (A_{520nm}-A_{700nm})_{pH4.5}; MW (molecular weight of malvidin-
200 3-glucoside = 493.43g/mol; DF = dilution factor; 1 = path length in cm; ϵ = 28000 molar
201 extinction coefficient and 10³ = factor for conversion from g to mg and cm.

202

203 2.8 Identification and Quantification of Anthocyanins by HPLC

204 The separation of the polyphenols was performed using an Agilent HPLC 1100 series
205 equipped with a degasser, a quaternary pump and a photodiode array detector model
206 (Agilent, Waldbronn, Germany) with Chemstation software. The column used was a C18
207 HiChrom (150 mm x 4.6 mm i.d; 5 μ m particle size and 100 Å pore size; part no.EXL-121-
208 1546U) operated at 30°C. The separation method was the same as described in our previous
209 paper (MohdMaidin et al., 2017). The polyphenols were monitored at 280nm and the UV/Vis

spectra were recorded in the range of 200 to 760nm. The main anthocyanins were detected at 520nm and identified based on the retention times and by comparing the spectra with that of the external standards which were: delphinidin-3-o-glucoside (>99%) (RT= 4.8; calibration curve $R^2 = 0.8771$); cyanidin-3-o-glucoside (>98%) (RT= 7.8; calibration curve $R^2=0.98744$); petunidin-3-o-glucoside (>98%) (RT= 8.5; calibration curve $R^2=0.99702$) and malvidin-3-o-glucoside (>99%) (RT= 7.8; calibration curve $R^2= 0.99994$); all supplied by Extrasynthese, Paris, France.

217

2.9 Determination of CIELab colour parameters and pH

The changes in colour of the EE, EE-SA and the CGA processed samples were measured using a CT-1100 ColourQuest HunterLab by taking the measurements in transmittance mode. Standard black plates were used for standardization. L^* , a^* and b^* measurements were obtained and used to calculate chroma and hue angles based on equations 4 and 5 below. Delta E (ΔE) was calculated based on the changes of the values of L^* , a^* and b^* at a given time, in comparison to these values at day 0 and applying equation 6.

$$\text{Hue angle } (^{\circ}) = \left[180 * \left(\frac{\text{ATAN}\left(\frac{b}{a}\right)}{\pi} \right) \right] + \left(\frac{b}{a} \right) \quad (\text{Equation 4})$$

$$\text{Chroma} = \sqrt{a^2 + b^2} \quad (\text{Equation 5})$$

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{\frac{1}{2}} \quad (\text{Equations 6})$$

229

230 The hue angle and chroma may be used on a CIE 1979 L*a*b* colourimetric system
231 diagram to identify colour and monitor changes. The changes of colour in all the CGA
232 processed samples over time were compared to EE and EE-SA as the control samples.

233 The pH of all samples was monitored regularly with a pH meter (Mettier-Toledo
234 SevenEasy), which was calibrated by using pH 4.0 and 7.0 buffer solutions (Sigma, UK).

235

236 2.10 Statistical analysis

237 All the experiments were performed in triplicate. The data were subjected to the
238 analysis of variance using IBM® SPSS® Statistics 21 software program where statistical
239 differences were noted. Differences among the different treatments were determined by
240 using the Tukey test. The significance level was defined at $p < 0.05$. The results were reported
241 as means \pm SD.

242

243 3. Results and Discussion

244 3.1. Changes of total phenolic content over storage time

245 Initial values for total phenolic content was measured in control samples (EE and EE-
246 SA) and three of the CGA processed samples at day 0. The total phenolic content for all
247 samples range was 285-2080 mg GAE/L. The TP content for EE-SA was higher ($p = 0.0371$)
248 than EE, which can possibly be explained by the presence of sorbic acid. The total
249 monomeric anthocyanin range was 99.1-422.9 mg ME/L. The antioxidant activity range was
250 2299-14469 μ M Trolox equivalent.

251 Over a storage period of 32 days, the losses in the TP content were minimal in all the
252 samples (Fig. 2A). The maximum degradation observed in EE-SA and EE was not more than
253 10%. Among all the CGA processed samples, the lowest losses of the TP content was in V16

(4.91%), followed by V8 (5.44%) and finally V4 (6.42%), although they were not significantly different ($p = 0.062$).

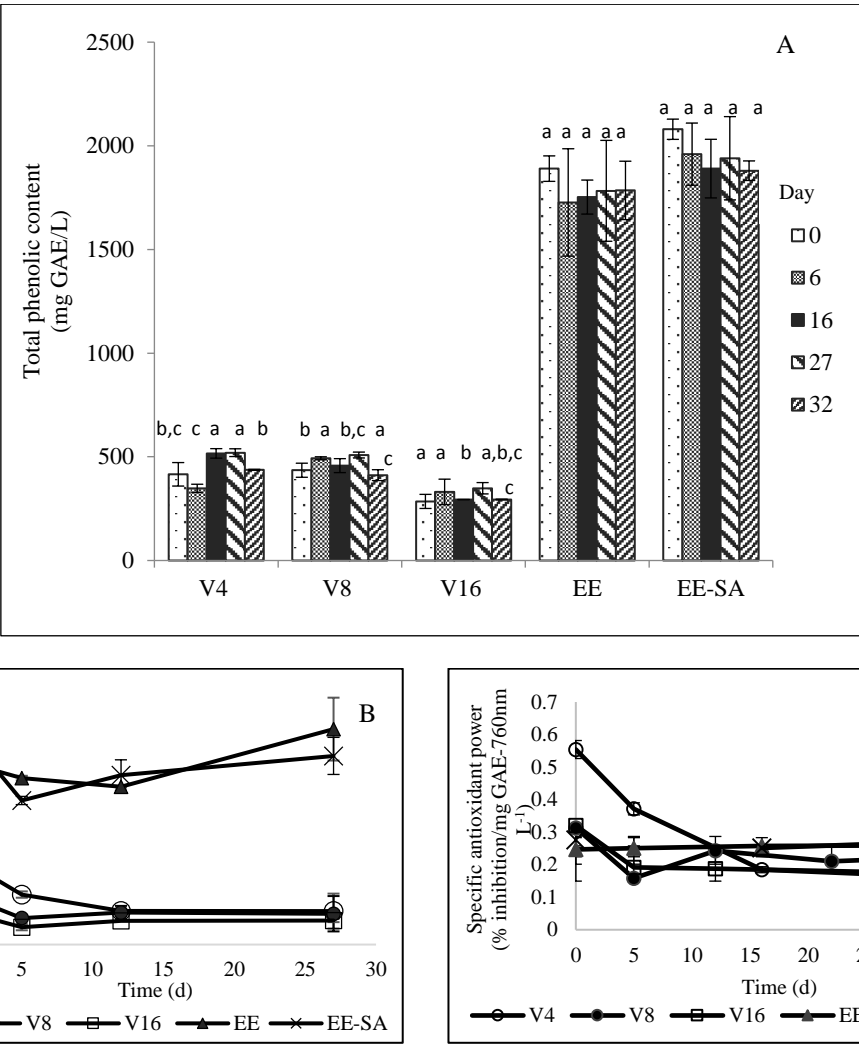


Figure 2. Total phenolic content (A), antioxidant activity profile expressed as Trolox Equivalent (μM) (B), and specific antioxidant power (% inhibition/mg GAE-760nm L⁻¹), (C) of CGA processed and control samples over time. Error bars represent means ± SD (n=3). V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated

264 at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.
265 Different letter within each series indicates significant differences using Tukey's test ($P < 0.05$).

266

267

268 3.2. *Changes in antioxidant activity over storage time*

269 The antioxidant activity of the control samples and CGA processed samples stored
270 over time was evaluated using ABTS assay. Figure 2 (B) shows a decrease in antioxidant
271 activity in both, the control samples and the CGA processed samples over time. The
272 reduction in antioxidant activity was clearly observed for all samples during the first 5 days.
273 Further decrease was observed in V4 after 5 days, however in V8, V16 and control samples
274 no further reductions or even slight increases in antioxidant activity was observed after this
275 time.

276 Moreover, when the specific antioxidant power (calculated as percentage of inhibition
277 per total phenol content) was calculated, a more distinct pattern was observed (Fig. 2C). In
278 general, the specific antioxidant power decreased over time at a higher rate than the total
279 phenols (Fig. 2A). Rapid loss of antioxidant power was observed particularly in V4 from
280 day 0 to day 16, and in V8, V16 and EE-SA only over the first 5 days. It was interesting to
281 note that there was no specific antioxidant loss in EE. This could be related to the total
282 phenolic content as depicted in Figure 2A where the losses in EE were not significantly
283 different ($p \leq 0.05$) between the time points. This implied that the losses in TP and antioxidant
284 activity in EE were in the same proportion hence the antioxidant efficiency was almost
285 constant over time (Fig. 2B). However, this was not the case for the CGA samples where
286 minimal losses of total phenolic content were noted but important changes in antioxidant
287 activity were observed. Therefore, this suggests that in these samples the antioxidant activity
288 may not be solely derived from the total phenolic content and/or that the phenolics undergo
289 some chemical changes that affect their antioxidant activity. Over estimation of total

phenolic content could possibly happen by the high sugar content or ascorbic acid in the crude extracts and the CGA processed samples (Ainsworth & Gillespie, 2007).

3.3. Kinetics of total anthocyanins degradation over storage time

Degradation of anthocyanins has been previously studied in wine and its residues (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Clemente & Galli, 2011, 2013; Lavelli et al., 2017). The patterns of degradation involving anthocyanins are complex, but the degradation rate generally follows first-order kinetics (Amendola, De Faveri, & Spigno, 2010; Buckow, Kastell, Terefe, & Versteeg, 2010). However, the information on anthocyanin degradation in the presence of surfactant is lacking. In this study, the degradation of anthocyanins in the control samples (EE and EE-SA) were compared with the CGA processed samples stored at 20°C, which also followed first-order kinetics (Fig. 3).

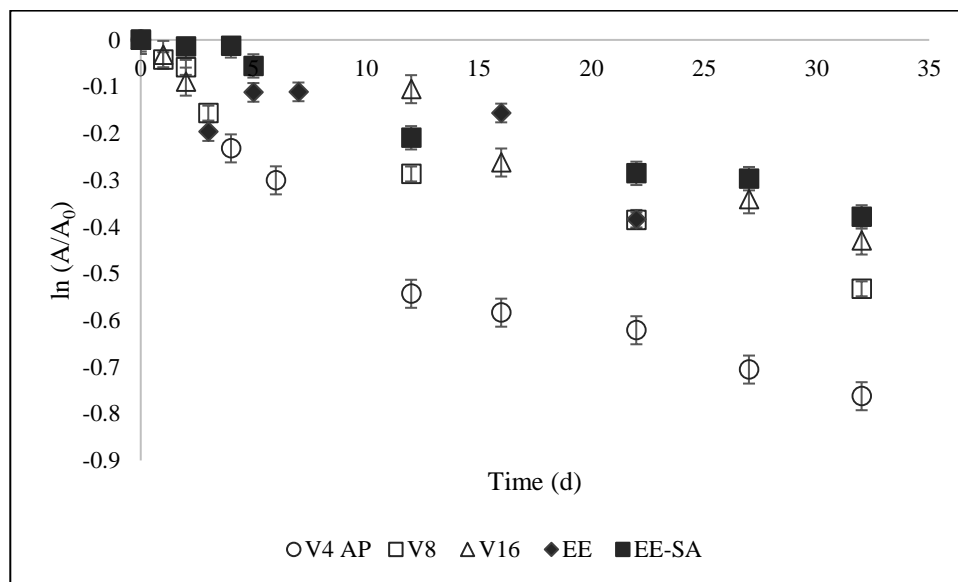


Figure 3. Time course for the decrease of anthocyanins represented here as the natural logarithm of the ratio of anthocyanins concentration at a given time and at time zero (A_0) during storage at 20°C. Error bars represent means \pm SD. V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

Degradation of anthocyanins in all samples followed first order kinetics as shown by data in Figure 3. In Table 1A the first-order rate constant (k) and the linear regression coefficient (R^2) of all samples are shown. The first-order rate constant for anthocyanins degradation ranged between 0.0124 and 0.0217 d⁻¹. Although the R^2 values for V4 and EE were lower than the others, first-order kinetics were assumed. This was also based on the assumption that since the pH of these samples ranged 3.5-3.8 the degradation of the anthocyanins and thus the ionization of flavillium ion followed first-order kinetics as found by West & Mauer (2013). The first-order rate constant of EE was higher ($k = 0.0159$ d⁻¹) than the one of EE-SA; the first-order rate constant of EE-SA was the lowest among all samples ($k = 0.0121$ d⁻¹). This suggests that the addition of sorbic acid increased the stability of anthocyanins during storage although sorbic acid has only been reported to prevent microbial growth during storage (Troller & Olsen, 1967).

As shown in Table 1A the first-order rate constant decreased with the increase in volumetric ratio and thus with an increase in surfactant content in the CGA fractions (ranging from 6.07-8.58mM TWEEN20). The samples with the highest stability were the EE-SA and the CGA fraction with the highest surfactant concentration, V16, followed by V8 and EE which had very similar stability. The sample with the lowest concentration of surfactant V4, was found to degrade the fastest over time.

The extraction of grape pomace with water containing 3% of citric acid has also been proposed to recover phenolic-rich coloured extracts with 36-62% of total anthocyanins composition (Cardona, Lee And, & Talcott, 2009). However, the colour degradation of these water-based extracts at 30°C is fast, with first-order rate constants of 0.0364 and 0.038 for cold and hot pressed extractions, respectively. In the present study, the first-order rate constants were lower indicating more stable extracts. The most stable sample was the EE-SA ($k = 0.0121$ d⁻¹) suggesting a stabilisation effect of sorbic acid. Comparable results were

333 obtained for V16 CGA with $k = 0.0124\text{d}^{-1}$. However, interestingly the stabilisation effect in
 334 the CGA processed samples was only achieved above a certain concentration of surfactant
 335 as in V4 and V8 CGA samples the observed stabilisation effect when compared against the
 336 EE sample was minimal. It was estimated that the surfactant concentration in V16, V8 and
 337 V4 was 8.58mM, 7.56mM and 6.07mM respectively (see Methods). Therefore the
 338 concentration of surfactant in the samples should be at or above 8.58mM (about 1%) in order
 339 to have a stabilisation effect.

340

Table 1A. First-order empirical rate constants (k) and half-life for anthocyanins.

Sample	R^2	$K (\text{d}^{-1})$	$t_{1/2} (\text{d})$	Loss (%)*
V4	0.8861	0.0217 ± 0.0019	31	53.35
V8	0.9585	0.0157 ± 0.0024	44	41.30
V16	0.9385	0.0124 ± 0.0015	55	34.90
EE	0.8131	0.0159 ± 0.0012	43	41.04
EE-SA	0.9583	0.0121 ± 0.0011	57	31.53

Rate constants are expressed as means \pm SD. V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

*anthocyanins loss calculated after day 32

Table 1B. Half-lives ($t_{1/2}$, day) and degradation rate (k , d^{-1}) of different anthocyanins in control and CGA samples, stored at 20°C

Compound/Sample	V4 $T_{1/2}/K$ (d/ d^{-1})	V8 $T_{1/2}/K$ (d/ d^{-1})	V16 $T_{1/2}/K$ (d/ d^{-1})	EE $T_{1/2}/K$ (d/ d^{-1})	EE-SA $T_{1/2}/K$ (d/ d^{-1})
Delphinidin 3-o-glucoside	35 0.0195	36 0.0190	29 0.0233	41 0.0168	41 0.0168
Cyanidin 3-o-glucoside	42 0.0163	55 0.0126	52 0.0132	33 0.0204	42 0.0162
Petunidin 3-o-glucoside	50 0.0136	44 0.0155	52 0.0132	50 0.0136	49 0.0139
Malvidin 3-o-glucoside	55 0.0126	57 0.0121	71 0.0097	59 0.0116	65 0.0116

341

342 A study conducted by Lavelli et al., (2017) in an ethanolic extract of grape pomace
343 maltodextrin-encapsulated showed a low first-order rate constant, $0.0033\text{--}0.0014\text{ d}^{-1}$. This
344 might be due to lower water activity content and therefore these results are not comparable
345 with the present results, as this study assessed the stability of anthocyanins in a liquid form.
346 Moreover, the drying process will require higher overhead costs, and needs high energy and
347 pressure input which would add up greatly to the overall cost.

348 The half-lives of anthocyanins of EE and EE-SA stored at 20°C were 43 and 57 d,
349 respectively (Table 1A). As discussed above, the degradation is faster in the CGA samples
350 with lowest surfactant concentration so V4 had the shortest half-life of 31 d, followed by V8
351 (44 d) and V16 (55 d). Moreover the half-life of V16 was almost similar to EE-SA's, but
352 longer than that of EE. These half-life values were higher than the ones reported for the
353 blueberry juice stored at 25°C ($t_{1/2} = 4.4\text{ d}$), possibly due to the different types of
354 anthocyanins present (Buckow et al., 2010). Similarly, when the percentage of anthocyanin
355 losses after 32 days of storage was determined, the EE-SA had the least loss (31.53%),
356 closely followed by V16 (34.90%).

357 In summary, from all the above data it can be concluded that the surfactant had a
358 stabilisation effect on anthocyanins and this effect was comparable to that observed in
359 extracts with sorbic acid. To the best of our knowledge, only one report by Thakur & Arya
360 (1989) assessed the stability of anthocyanins in grape juice preserved with sorbic acid and
361 their result agreed with the findings in this study. This further confirmed that the surfactant
362 might play an important role in protecting the anthocyanins from oxidation, thus extending
363 the half-life.

364

365 3.4. Anthocyanins Identification and Quantification

366 The HPLC-DAD analysis showed that all samples had 13 anthocyanins identified at
367 the beginning and at the end of storage study (Figure S1), which was in agreement with our
368 previous study (MohdMaidin et al., 2017). The identified anthocyanins were: delphinidin,
369 cyanidin, petunidin, peonidin and malvidin with different glycosyl acylation attached. In red
370 wines and their pomace made from *V. vinifera* grapes, the main anthocyanins detected were
371 of 3-o-monoglucosides of the free anthocyanidins including pelargonidin-3-o-glucoside,
372 cyanidin-3-o-glucoside, delphinidin-3-o-glucoside, peonidin-3-o-glucoside, petunidin-3-o-
373 glucoside and malvidin-3-o-glucoside (Drosou et al., 2015; He et al., 2012; Kammerer et al.,
374 2004). However, in this study, pelargonidin-3-o-glucoside was not detected and four
375 anthocyanins (malvidin 3-o-glucoside, cyanidin 3-o-glucoside, delphinidin-3-o-glucoside,
376 and petunidin-3-o-glucoside) were quantified as these are the most abundant anthocyanins
377 present.

378 In all the samples, the most abundant anthocyanin was malvidin-3-o-glucoside
379 (0.68mg/g) which was in agreement with other studies (Bimpilas, Panagopoulou,
380 Tsimogiannis, & Oreopoulou, 2016; Morais, Ramos, Forgács, Cserhádi, & Oliviera, 2002)
381 followed by delphinidin-3-o-glucoside (0.58mg/g). Both pigments were typically responsible
382 for the purple and purple-blue which could be seen in the colour of the ethanolic extract.

383 Different anthocyanins had different degradation kinetics in each sample (Table 1B).
384 Among the four anthocyanins, delphinidin was the least stable anthocyanin in all the samples
385 except in EE. This can be seen in their short half-lives (29-41d). Fleschhut, Kratzer,
386 Rechkemmer, & Kulling (2006) reported that an increase in hydroxyl groups in the B ring
387 of the anthocyanin nucleus could result in a decrease in the stability which could possibly
388 account for the anthocyanins loss. However, this was not observed in EE where cyanidin
389 degraded faster than delphinidin, but both of them seemed to be less stable than petunidin

390 and malvidin indicating that methylation of hydroxyl-groups in B ring increased the stability
391 of anthocyanins. Our results were comparable to those reported by Hellstrom et al., (2013)
392 for delphinidin and cyanidin in the blackcurrant and chokeberry juices stored at 21°C with
393 half-lives between 16-44 days.

394 Malvidins are known to be the most stable as compared to other anthocyanins due to
395 the absence of two hydroxyl groups in the B ring structure. This was clearly evident as they
396 had the longest half-life as compared to other anthocyanins across all samples. Interestingly,
397 malvidin in V16 sample had longer half-life (71d), with slowest degradation rate ($k = 0.0097$
398 d^{-1}) than any of the control samples including EE-SA ($k = 0.0116\text{d}^{-1}$, $t_{1/2} = 65\text{d}$) which agrees
399 with the above observation on the protecting effect of the surfactant.

400 According to Hellström et al., (2013), the effect of the sugar moiety was minor as
401 compared to the effect induced by the type of the core anthocyanidin. In these extracts,
402 malvidin- and delphinidin 3-glucosides were the two predominant anthocyanins.
403 Delphinidin glucosides exhibited greater temperature sensitivity due to their three hydroxyl
404 group in the B ring in comparison to malvidin derivative which had only one –OH group
405 attached to it (Buckow et al., 2010). This can be clearly seen in the half-life of malvidin-3-
406 glucoside ($t_{1/2} = 55\text{-}71$ days) across all samples. Moreover, the stability of anthocyanins was
407 also reduced by the number of hydroxyl groups in the A ring with the absence of dihydroxyl
408 group in the B ring (Buckow et al., 2010). The matrix of samples also has been reported to
409 have a major impact on the stability of anthocyanins where anthocyanins in juices were more
410 prone to degradation as compared to those in smoothies, where the anthocyanins may be
411 protected by other phenolic compounds but the concrete reasons of this impact remained
412 unclear (Hellström et al., 2013). A study on anthocyanins stability from encapsulated grape
413 skin showed significant increase in the half-life of anthocyanins up to 452 days. The study
414 also proved that lowering the water activity of the encapsulated grape skin powder can

double the half-life up to 998 days (Lavelli et al., 2017). In fruit juices, several factors can influence the stability of anthocyanins, such as pH, presence of ascorbic acid and anthocyanin degrading enzymes (Buckow et al., 2010). Finally, the degree of glycosylation also might possibly affect anthocyanins stability; the higher the degree of glycosylation, the more stable they became.

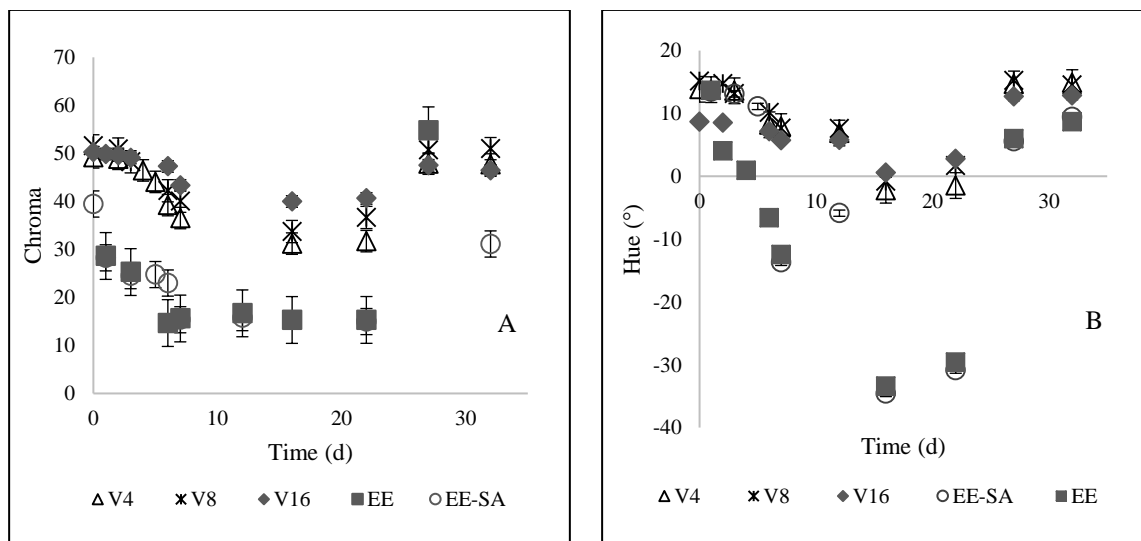
Co-pigmentation of anthocyanins with other compounds is considered as an important mechanism of colour stabilisation in plants. Anthocyanins can form co-pigments with metal ions, other phenolic compounds or through self- association (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Co-pigmentation through self-association is unlikely because in order for it to take part, the concentration of the anthocyanin should be greater than 1mmol/L. Hydroxycinnamic acids and flavonols were reported as the best cofactors in wine (Bimpilas et al., 2016). Co-pigmentation can be influenced by the anthocyanins and co-pigment structure, and also by the concentration of anthocyanins to the co-pigment (Eiro & Heinonen, 2002). In the present study, the ratio of anthocyanins and co-pigments might not be sufficient for the co-pigmentation to occur since no additional phenolic acids were added to the samples. Thus, the stabilisation effect observed in this study was solely due to the surfactant and the addition of sorbic acid.

3.5. *Colour stability and pH*

Grape pomace extract had high levels of anthocyanins. However, anthocyanins undergo degradation during processing and storage, thus affecting colour characteristics. In the present study, the colour stability of the control samples and the CGA processed samples was investigated and compared against EE and EE-SA. Colourimetric parameters chroma (C) and hue (h) and ΔE were determined to assess colour changes over time. The effects were comparable to those observed in anthocyanins, yet with some exceptions.

Figure 4 (A and B) showed the changes of colour in chroma and hue angle for the control samples and the CGA processed samples over time. The results obtained showed that EE and EE-SA had a similar trend; ΔE values overlapped with each other. At day 0, both samples had dark red colour ($c = 39.4$, hue = 6.9). Over time, both chroma and hue values decreased rapidly by day 22, from dark red tending towards blue-black shade ($c = 14.9$, hue = -29.6).

The same trend was observed in all of the CGA processed samples, although the chroma and hue angles decreased steadily as compared to EE and EE-SA. At day 0, all of the CGA processed samples had almost similar colour of dark red shade ($c = 49.2$ -51.5; hue = 8.7-15.1). However, the chroma ($c = 31.7$ -40.6) and hue angle (hue = -1.4-2.8) values decreased in all of the CGA processed samples over time. In short, V4 turned from dark red to light red, tending towards blackish; V8 turned from dark red to light red, tending towards browning and finally, V16 turned from dark red to light red, tending towards pinkish. Therefore, these results showed that minimum colour changes were observed in V16, which correlated with the lower degradation rate of anthocyanins determined above and confirms the stabilisation effect by the surfactant.



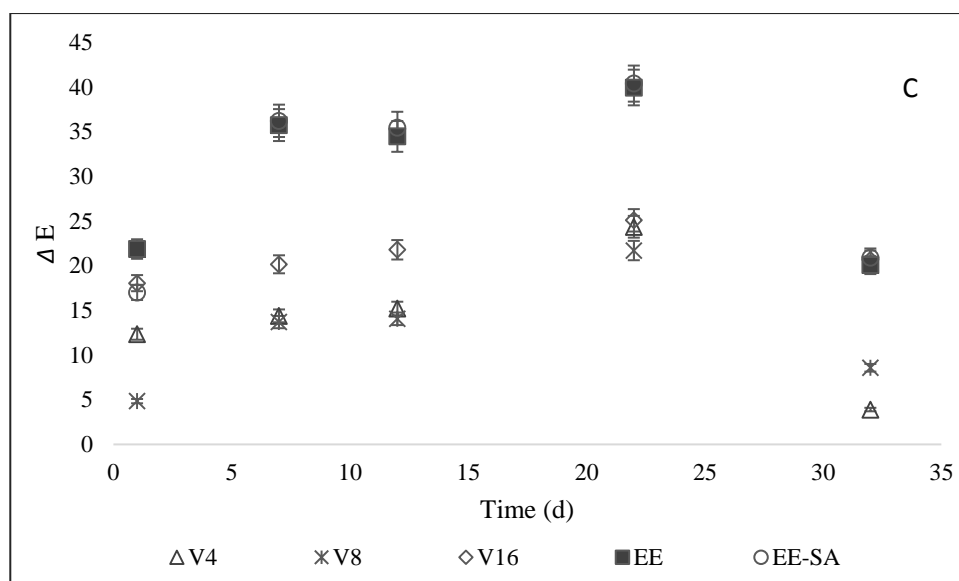


Figure 4. Chroma and hue values of samples during storage (A and B), total colour difference (ΔE) between samples (C). Error bars represent means \pm SD ($n = 3$). V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

460

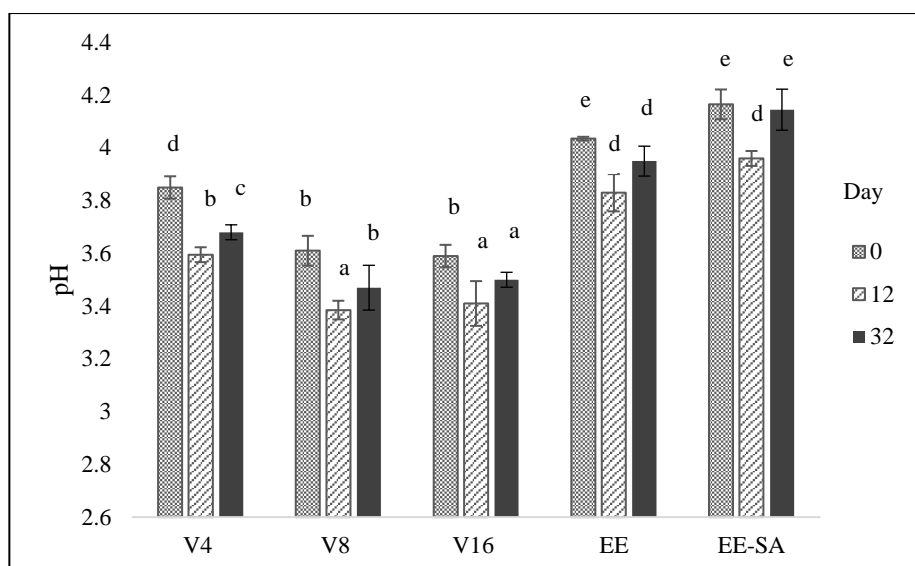
461 Furthermore, the colour changes of samples can be further explained by ΔE (Fig. 4C).

462 ΔE indicates the magnitude of the colour difference between fresh and stored grape extracts
 463 for different time points. Higher colour differences were measured for the control samples
 464 than for the CGA samples. In all the CGA processed samples, the changes were not
 465 significantly different ($p > 0.05$) in the first 12 days of storage. However, higher magnitude
 466 of changes was observed in day 22, and minimal changes were observed in day 32.

467 Anthocyanins differ from each other by the number and position of the hydroxyl, and
 468 methoxyl substituent groups in the B ring of the molecule. The hydroxylation pattern of the
 469 anthocyanins in the B ring can directly affect the hue and colour stability due to the effect
 470 on the delocalized electron path length in the molecule (He et al., 2012). Anthocyanins with
 471 more hydroxyl groups in the B ring can contribute more to blueness, meanwhile the degree
 472 of methylation in the B ring can increase redness. The rapid decrease in red colour of EE
 473 and EE-SA might be explained by the degradation of a particular anthocyanin. In both

control samples, cyanidin-3-glucoside and petunidin-3-glucoside had the shortest half-lives between 16-21 days (Table 1B). Both anthocyanins were responsible for the red and dark red colour respectively, which could explain the losses of dark red colour in both controlled samples after 21 days. Both anthocyanins had two hydroxyl groups attached to the B ring, which increased the blueness of the colour, as found in these samples. In the case of the CGA processed samples, V4 appeared to have the same result as EE and EE-SA, which is supported by the short half-life of cyanidin-3-glucoside determined in this sample. However, in the case of V8 and V16, delphinidin-3-glucoside had the shortest half-life, 31 and 36 days respectively. This could have contributed to the colour changes observed, from dark red to light red, tending towards brownish and pinkish. Delphinidin-3-glucoside is responsible for the blueness as it has three hydroxyl groups attached to the B ring.

Although most studies showed that delphinidin-3-glucoside exhibited a greater thermal sensitivity due to their three hydroxyl substitution group, this was not clearly observed in this study; thus the correlation between anthocyanin stability and chemical structure is still unclear (Rice-Evans, Miller, & Paganga, 1996). Moreover, the colour changes in EE-SA could not be explained by the slowest anthocyanins loss in this sample. This suggests that the mechanism of colour stabilisation in this sample needs further study as colour change does not correlate with anthocyanins degradation. The mechanisms of stabilisation of anthocyanins by TWEEN20 are yet to be determined, but we propose that the micelles might play a role in encapsulating the anthocyanins protecting them against oxidation during storage.



495

496 Figure 5. pH values of all samples on selected days. Error bars represent means \pm SD, n = 3. V4,
 497 CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16,
 498 CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic
 499 extract with sorbic acid.

500

501 Overall, the pH of all samples dropped and then increased slightly (Fig. 5). Although
 502 it is known that pH plays an important role in determining the state of the flavylum ion, the
 503 trend was unclear in the present study. This could possibly be due to the presence of
 504 TWEEN20 which could have a stabilisation effect as shown by the reduction of pH after
 505 CGA separation. The increased in pH values later throughout the end of storage may be due
 506 to the formation of phenolic acids like gallic acid; this can be supported by the fluctuations
 507 in total phenolic content over storage which may be also an indication of the formation of
 508 these intermediate compounds (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010).

509

510 4. Conclusions

511 The stability of the ethanolic raw extract from grape marc was compared with that of
 512 the further processed sample after applying the CGA separation. The main effect was found
 513 on the stability of anthocyanins. Anthocyanins stability in CGA fraction V16 was higher

514 than in the raw extract based on the comparison of the first order kinetics of anthocyanins
515 degradation followed by all the samples. The stability in CGA samples increased with an
516 increase in surfactant concentration, V16 sample having the highest half-life (55 d) and
517 similar to the raw extract's with sorbic acid (57 d). Thus these results show that the surfactant
518 has a stabilization effect on the anthocyanins and the sorbic acid seems to have a similar
519 effect. Moreover a good correlation between the colour changes and degradation rate of
520 individual anthocyanins was observed whereby malvidin-3-o-glucoside was found to be the
521 most stable anthocyanin at all the studied conditions with the highest half-life found in V16.
522 Overall, this study shows that the surfactant has a stabilisation effect on the anthocyanins
523 and half-lives determined here were higher than others reported for wet formulations of
524 anthocyanins. The mechanism of stabilisation of anthocyanins by TWEEN20 may be related
525 to the solubilisation of the anthocyanins within the micelles. Furthermore, the main findings
526 in this study have shown the advantages of CGA as a separation method that can also
527 integrate a pre-formulation step.

528

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533

534 **Conflict of interest**

535 The authors declare no conflict of interest.

536

537

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